Atty. Dkt. No.:EPI3007D
(formerly TSRI 184.2CON-3)

REMARKS

Claims 43, 48, 50, 58-63 and 65-67 are currently pending following entry of the instant Amendment. The amendment results in a net reduction of 31 claims, leaving claim 43 as the sole only a single independent claim. As presently constituted, the claimed invention is directed to a plant cell that contains nucleotide sequences encoding a biologically functional multimeric protein which is an immunoglobulin molecule. The plant contains nucleotide sequences encoding an immunoglobulin heavy and light chain polypeptide wherein each polypeptide contains a leader sequence forming a secretion signal. The plant cell also contains immunoglobulin molecules encoded by the nucleotide sequences, wherein each leader sequence forms a secretion signal that is cleaved from the immunoglobulin heavy chain and light chain polypeptide following proteolytic processing.

The amended claims and the newly added claims are fully supported by the specification, and do not introduce new matter or require new search. Support for claim 43 can be found, for example, at page 73-77 (Examples 7 and 8). Applicants respectfully submit that the amendments should be entered into the case because they either place the case in condition for an allowance or reduce issues upon appeal.

The examiner's attention is directed to the fact that the sole independent claim remaining in the case (claim 43) now contains language similar to claims that the examiner has previously indicated are allowable over the same subject matter (Düring and Goodman) in copending U.S. Serial No. 09/199,534 (compare instant claim 43 to claim 21 in 09/199,534). In view of these amendments and arguments below, Applicants respectfully submit that the application is in condition for allowance.

AMENDMENT OF THE SPECIFICATION

Applicants have amended the cross reference section in the first paragraph of the specification, as noted above. Accordingly, the status of all parent priority applications/patents have been updated.

Atty. Dkt. No.:EPI3007D
(formerly TSRI 184.2CON-3)

NON-STATUTORY DOUBLE PATENTING

Claims 21-64 and 65-68 have been provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 21-30 and 36-37 of copending Application No. 09/199,534;

Claims 21-40, 42-64 and 65-68 have been provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 21 and 32-78 of copending Application No. 09/200,657;

Claims 21-40, 42-64 and 65-68 have been rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 6-12 of U.S. Patent No. 5,959,177;

Claims 21-40, 42-64 and 65-68 have been rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 1-5 of U.S. Patent No. 5,202,422; and

Claims 21-40, 42-64 and 65-68 have been rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 1-7 of U.S. Patent No. 5,639,947.

As discussed in the previous response (Paper No. 8, pages 4-6), Applicants respectfully disagree and traverse the rejection. In the interests of furthering prosecution of the case, however, the above mentioned disclaimers have been provided herewith. Applicants reserve the right to later withdraw the disclaimer depending on circumstances.

REJECTION UNDER 35 U.S.C. § 102 OVER DÜRING

The rejection of claims 21-40 and 42-68 under 35 U.S.C. § 102(b) as being allegedly anticipated by Düring (Dissertation) is respectfully traversed. Claims 21-40, 42, 44-47, 49, 51-57, 64, and 68 have been cancelled herein, rendering the rejection moot as to these claims. Reference to page numbers in During are to th English language translation.

Relevant Law

In order to anticipate a claim, a single prior art publication must provide each and every element set forth in the claim. Furthermore, the claims must be interpreted in light of the teachings of the specification. *In re Bond*, 15 USPQ2d 1566, 1567 (Fed. Cir. 1990). *See* also MPEP §2131.

Argument

The claims as amended herein are directed to a plant cell that contains nucleotide sequences encoding an immunoglobulin heavy and light chain polypeptide wherein each polypeptide contains a leader sequence forming a secretion signal. Also required is that the plant cell contain biologically functional immunoglobulin molecules encoded by the nucleotide sequences, wherein each leader sequence forms a secretion signal that is cleaved from the immunoglobulin heavy chain and light chain polypeptide following proteolytic processing. Thus, as presently constituted, the claimed invention requires that the heavy and light chain of the immunoglobulin contain a leader sequence which is removed during proteolytic processing.

Although Applicant believes that the prior pending claims are patentably distinct over the cited art, in the interests of furthering prosecution, claim 43, the sole remaining independent claim in this case, now contains language similar to claim 21 of copending 09/199,534, the latter previously deemed allowable by Examiner Phuong Bui over Düring and/or Goodman. As was argued successfully in copending 09/199,534, neither Düring or Goodman, alone or in combination fail to anticipate or render obvious a plant cell that contains nucleic acid sequence encoding an immunoglobulin light and heavy chain each with a leader sequence and contains immunoglobulin molecules where the leader sequences are removed following proteolytic processing in the cell.

The claimed invention includes nucleic acid sequences which encode an immunoglobulin polypeptide that enters into the protein secretory pathway once the leader sequences are cleaved. This strategy requires that the leader sequence is located directly in front of (5′ to) the DNA encoding the mature polypeptid . Correct processing of the

protein allows the assembly and secretion of the immunoglobulin protein and accumulation in the Golgi vesicles and the cell wall. The During dissertation discloses a distinctly different strategy. In this case, the barley amylase leader sequence was cloned directly in front of (5' to) the heavy chain polypeptide encoding DNA while in the case of the light chain additional amino acids were placed between the barley leader and the light chain polypeptide encoding DNA (i.e., Gly-Ser-Met). During, p.18 at top of page. As detailed in the dissertation, the alleged resulting immunoglobulin molecules were not detected in the cell wall or golgi but were detected only in chloroplasts and in the cytoplasm. This indicates that the molecules the light chain leader sequence was not processed correctly affecting assembly of the molecule and its entry into protein secretory pathway. *Id.* During, therefore, chose two distinct strategies for expressing each immunoglobulin chain.

The additional amino acids that would be encoded at the 3' end of the light chain leader sequence constructed by During were unusual in the context of known eukaryotic signal cleavage sites. At the time of During's Dissertation and prior to filing the present application, it was not clear what effect additional amino acids at the end of a leader sequence would have on final processing of the leader. Studies by the present Applicants as well as others in the art indicate that by introducing Gly-Ser-Met amino acids between the C-terminal end of the leader and the first amino acid of the mature kappa chain, the structure of the potential cleavage site is altered.

Thus, During teaches an alternative strategy to that of the claimed invention where the expression of the light chain is directed by a chimeric signal peptide-light chain gene which includes three amino acids not normally found at the C-terminus of a eukaryotic signal sequence (During, p. 18, line 7; Figure III/4). Using this strategy, Düring detected "aggregated" immunoglobulin as defined by immunoreactivity with AC38 in the cytoplasm and principally in chloroplasts, while no immunoglobulin was detected in the cell wall, golgi or vesicles. During, p94. During's results are at odds with protein secretion in plants and were in fact at odds with his own data on expressing lysozyme in plants where the protein leader sequence was not mutated and the single polypeptide processed and entered the secretory pathway and eventually was detected in the cell wall but not in chloroplasts. During, p.97, lines 6-23.

During, therefore, used a different strategy from the present inventors and achieved a transgenic plant that does not result in correct post-expression processing and secretion of the heavy and light chain resulting in a complete and fully immunoglobulin molecule. On this basis alone, During fails to anticipate the claimed invention.

Applicant further respectfully submits that During is not an enabling disclosure because there are numerous deficiencies which would raise reasonable doubt that Düring even succeeded in expressing and immunoglobulin in a tobacco plant. Düring was unable to detect immunoglobulin light chain expression following transfection of plant cells with a vector encoding only a light chain gene. Düring, p. 80, line 2 ("Repeated attempts to directly detect the light chain of B1-8 and for T4 lysozyme from the crude extract of tobacco mesophyll protoplasts were unsuccessful."). Because it was known for mammalian cells that light chain was much easier to express than heavy chain, Düring's failure to express light chain would have raised serious doubts coloring the reasonable believability of his ultimate claims to have secreted a biologically active complete heavy-light chain antibody.

Despite the failure to achieve light chain expression by itself, Düring proceeds to clone the heavy chain into the expression vector and attempts immunoglobulin expression with both a light and a heavy chain in plant cells. Convinced that he could not directly detect immunoglobulin expressed by his plant cells, Düring attempts to develop a method to enrich the antibody from extract before performing detection by Western blotting. During, p87, 3rd and 4th paragraph.

To enrich for antibody in the plant extract, Düring exposed large volumes of plant the extract to several rounds of affinity purification with CNBr activated Sepharose 4b to which is attached Ls136 antibody (monoclonal antibody to light chain) and NP hapten. During, p. 87, 4th full paragraph through page 89, lines 11-13.

Düring reports that direct Western blotting (without affinity enrichment) resulted in detection of the light chain but not the heavy chain. Düring Thesis, page 89 lines 3-4 ("Only the detection of processed light chain was possible for different callus material."). Following processing to affinity purify antibody from the plant extracts, Düring again

detects only a light chain signal and infers that whole active antibody must have been present for such result to have been obtained.

> By using affinity chromatorgraphic purification with NP Sepharose and then Western blotting, the presence of a functional aggregated antibody to be indirectly demonstrated from induced leaf material of a Greenhouse A10 plant indirectly by detection of the light chain from isolated, biologically active hapten-bound plant B1-8 was determined.

Id. Düring also employs tissue printing which involves pressing the leaf against a membrane and attempting to detect the protein expression by reaction with the membrane. Düring, paragraph bridging p88 and 89. Düring now claims positive signal for both the light chain and the heavy chain and binding by the Ac38 antibody, which allegedly binds only when light chain is associated with heavy chain. Ac38 is a monoclonal antibody whose epitope is formed from both the heavy and light chain assemble in the correct tertiary and quaternary structure. However, the specificity of the Ac38 antibody used by During was never properly uncharacterized for the various assay formats used. Furthermore, there is no published report demonstrating that Ac38 can detect B 1-8 following the fixation and denaturation procedures used by During. During emphasizes that the Ac 38 antibody requires the correct tertiary and quaternary procedure to bind B 1-8 (During, p. 86), raising a question whether binding and/or specificity for the B1-8 epitope is present following a denaturation step. The deficiencies in the characterization of the AC38 antibody used by Düring would have raised serious doubts about the reasonableness of Düring's conclusions for successful antibody expression in plants.

To recount, when Düring attempts to express only the light chain, he detects nothing but when he express both the light and heavy together, he detects all chains by direct tissue printing and detects only the light chain (but infers the presence of an assembled light and heavy chain) by Western blotting of plant extracts which have been pre-enriched for antibody by affinity chromatography. Although differences between assays can under certain circumstances be adequately explained, when one encounters the situation described by Düring, there needs to be rigorous controls in place, particularly when one is working at the level of detection that Düring himself admits to be working.

Although Düring uses controls, he does not use a sufficient number of controls or adequately describe those few that he uses to allow proper scrutiny of the results. For example, in the tissue printing experiments, the only control used is described as follows:

> By saturation of the employed antibodies as wild type extract, no cross reactions develop for the control spot and the green color of the chlorophyll remained visible.

This description is wholly inadequate to explain the nature of the control. In any event, even if explained, more controls would be needed to allow the conclusion that Düring takes from the results. For example, the specificity of binding should have been evaluated by determining if it was subject to inhibition in the presence of purified immunoglobulin, purified heavy chain, purified light chain and other protein controls. It is inexplicable why Düring fails to use adequate controls because they were well known to those of ordinary skill in the art at the time of Düring. In addition, Düring's use of plant extract pre-enrichment prior to Western blotting also was inadequately described and one cannot discern whether he adequately controlled for artifacts, well known to exist with such a procedure. For example, it was well known CNBr linkage is subject to leaching Düring affinity chromatography, and that this varies with the agent used to remove the bound protein. See Goldenberg et al. Bioconjug Chem 1991 Jul-Aug;2(4):275-80 (Abstract attached as Exhibit A); Lihme et al., J Chromatogr 1986 Apr 11;376:299-305 (Abstract attached as Exhibit B). Düring, however, does not describe how protein was eluted from his Sepharose 4b absorbent. Düring translation, p. 88, end of first full paragraph ("After overnight incubation of the corresponding affinity gel at 4(C on the Denley roller, the bound protein is eluted in an Eppendorf flask under appropriate conditions and concentrated by ultrafiltration"). Furthermore, there is no indication in Düring that a control for column leaching was used or even considered. In addition, as already discussed, Düring failed to adequately characterize the specificity of the AC38 antibody in the assay format for which it was used.

Accordingly, because During fails to disclose each and every element of the claimed invention, and alternatively is a non-enabling disclosure, the rejection under §102(b) fails as

a matter of law. The examiner, therefore, is respectfully urged to withdraw the rejection of the claims.

REJECTION UNDER 35 U.S.C. § 102 OVER GOODMAN

The rejection of claims 21-40 and 42-68 under 35 U.S.C. § 102(e) as being allegedly anticipated by Goodman (U.S. 4,956,282) is respectfully traversed. Claims 21-40, 42, 44-47, 49, 51-57, 64, and 68 have been cancelled herein, rendering the rejection moot as to these claims. As already discussed the claims as amended herein are directed to a plant cell that contains nucleotide sequences encoding an immunoglobulin heavy and light chain polypeptide wherein each polypeptide contains a leader sequence forming a secretion signal. Also required is that the plant cell contain biologically functional immunoglobulin molecules encoded by the nucleotide sequences, wherein each leader sequence forms a secretion signal that is cleaved from the immunoglobulin heavy chain and light chain polypeptide following proteolytic processing.

Thus as presently constituted, the claimed invention requires that a secretion signal be used for both the heavy and light chain of the immunoglobulin and that there be proper assembly of the heavy and light chain following proteolytic processing which removes the leader from each chain. Goodman's teachings at best are limited to expressing gamma interferon, a lymphokine that is a single polypeptide and is functionally distinct from immunoglobulin. With only a single sentence that mentions the production of immunoglobulins, the Goodman disclosure is not enabling for immunoglobulin. Goodman's passing reference to immunoglobulin expression is mere speculation, amounting to nothing more than an invitation to experiment. Accordingly, because Goodman fails to disclose each and every element of the claimed invention, the rejection under §102(e) fails as a matter of law. The examiner, therefore, is respectfully urged to withdraw the rejection of the claims.

REJECTION UNDER 35 U.S.C. § 103 OVER DÜRING

The rejection of claims 21-40 and 42-68 under 35 U.S.C. § 103(a) as being allegedly obvious over Düring is respectfully traversed. Claims 21-40, 42, 44-47, 49, 51-57, 64, and 68 have been cancelled herein, rendering the rejection moot as to these claims.

Relevant Law

A claimed invention is obvious if the differences between it and the prior art "are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art." 35 U.S.C. § 103 (1994); see also Graham v. John Deere, 383 U.S. 1, 13 (1966).

Federal Circuit case law provides that "[t]he consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success, viewed in the light of the prior art." In re Dow Chem., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed.Cir.1988). Under the law, there must be a showing of a suggestion, teaching, or motivation to combine the prior art references is an "essential evidentiary component of an obviousness holding." C.R. Bard, Inc. v. M3 Sys. Inc., 157 F.3d 1340, 1352, 48 USPQ2d 1225, 1232 (Fed.Cir.1998). Also required is that the combined teachings have a reasonable expectation of success, viewed in light of the prior art. *See* In re Dow Chemical Co., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed.Cir.1988)("Both the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure.").

The examiner bears the burden of establishing a *prima facie* case of obviousness. In re Rijckaert, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed.Cir.1993); In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed.Cir.1992). This showing must be clear and particular, and broad conclusory statements about the teaching of multiple references, standing alone, are not "evidence." *See* Dembiczak, 175 F.3d at 1000, 50 USPQ2d at 1617. However, the suggestion to combine need not be express and "may come from the prior art, as filtered through the knowledge of one skilled in the art." Motorola, Inc. v. Interdigital Technology Corp., 121 F.3d 1461, 1472, 43 USPQ2d 1481, 1489 (Fed.Cir.1997). Only when the examiner's burden is met does the burden of coming forward with rebuttal argument or evidence shift to applicant. Rijckaert, 9 F.3d at 1532, 28 USPQ2d at 1956.

Argument

As already discussed under anticipation, the Düring reference fails to teach each and every limitation of the claimed invention. In particular, because the strategy used by Düring for expression is different from Applicants' claimed invention, resulting in expression that fails to achieve correct post-expression processing of the heavy and light chain resulting in a complete and fully immunoglobulin molecule. As noted by the Applicants, Düring's strategy likely obscured the recognition site for proteolytic processing and removal of the leader peptide, a step that is necessary for proper assembly of a heavy-light chain antibody construct.

Applicant further respectfully submits that During is not an enabling disclosure because it has numerous deficiencies which would raise reasonable doubt that Düring even succeeded in expressing and immunoglobulin in a tobacco plant. Düring was unable to detect immunoglobulin light chain expression following transfection of plant cells with a vector encoding only a light chain gene. Düring, p. 80, line 2 ("Repeated attempts to directly detect the light chain of B1-8 and for T4 lysozyme from the crude extract of tobacco mesophyll protoplasts were unsuccessful."). Because it was known for mammalian cells that light chain was much easier to express than heavy chain, Düring's failure to express light chain would have raised serious doubts coloring the reasonable believability of his ultimate claims to have secreted a biologically active complete heavy-light chain antibody.

Despite the failure to achieve light chain expression by itself, Düring proceeds to clone the heavy chain into the expression vector and attempts immunoglobulin expression with both a light and a heavy chain in plant cells. Convinced that he could not directly detect immunoglobulin expressed by his plant cells, Düring attempts to develop a method to enrich the antibody from extract before performing detection by Western blotting. During, p87, 3rd and 4th paragraph.

To enrich for antibody in the plant extract, Düring exposed large volumes of plant the extract to several rounds of affinity purification with CNBr activated Sepharose 4b to

which is attached Ls136 antibody (monoclonal antibody to light chain) and NP hapten. During, p. 87, 4th full paragraph through page 89, lines 11-13.

Düring reports that direct Western blotting (without affinity enrichment) resulted in detection of the light chain but not the heavy chain. Düring Thesis, page 89 lines 3-4 ("Only the detection of processed light chain was possible for different callus material."). Following processing to affinity purify antibody from the plant extracts, Düring again detects only a light chain signal and infers that whole active antibody must have been present for such result to have been obtained.

> By using affinity chromatorgraphic purification with NP Sepharose and then Western blotting, the presence of a functional aggregated antibody to be indirectly demonstrated from induced leaf material of a Greenhouse A10 plant indirectly by detection of the light chain from isolated, biologically active hapten-bound plant B1-8 was determined.

Id. Düring also employs tissue printing which involves pressing the leaf against a membrane and attempting to detect the protein expression by reaction with the membrane. Düring, paragraph bridging p88 and 89. Düring now claims positive signal for both the light chain and the heavy chain and binding by the Ac38 antibody, which allegedly binds only when light chain is associated with heavy chain. Ac38 is a monoclonal antibody whose epitope is formed from both the heavy and light chain assemble in the correct tertiary and quaternary structure. However, the specificity of the Ac38 antibody used by During was never properly uncharacterized for the various assay formats used. Furthermore, there is no published report demonstrating that Ac38 can detect B 1-8 following the fixation and denaturation procedures used by During. During emphasizes that the Ac 38 antibody requires the correct tertiary and quaternary procedure to bind B 1-8 (Düring, p. 86), raising a question whether binding and/or specificity for the B1-8 epitope is present following a denaturation step. The deficiencies in the characterization of the AC38 antibody used by Düring would have raised serious doubts about the reasonableness of Düring's conclusions for successful antibody expression in plants.

To recount, when Düring attempts to express only the light chain, he detects nothing but when he express both the light and heavy together, he detects all chains by

direct tissue printing and detects only the light chain (but infers the presence of an assembled light and heavy chain) by Western blotting of plant extracts which have been pre-enriched for antibody by affinity chromatography. Although differences between assays can under certain circumstances be adequately explained, when one encounters the situation described by Düring, there needs to be rigorous controls in place, particularly when one is working at the level of detection that Düring himself admits to be working.

Although Düring uses controls, he does not use a sufficient number of controls or adequately describe those few that he uses to allow proper scrutiny of the results. For example, in the tissue printing experiments, the only control used is described as follows:

> By saturation of the employed antibodies as wild type extract, no cross reactions develop for the control spot and the green color of the chlorophyll remained visible.

This description is wholly inadequate to explain the nature of the control. In any event, even if explained, more controls would be needed to allow the conclusion that Düring takes from the results. For example, the specificity of binding should have been evaluated by determining if it was subject to inhibition in the presence of purified immunoglobulin, purified heavy chain, purified light chain and other protein controls. It is inexplicable why Düring fails to use adequate controls because they were well known to those of ordinary skill in the art at the time of Düring. In addition, Düring's use of plant extract pre-enrichment prior to Western blotting also was inadequately described and one cannot discern whether he adequately controlled for artifacts, well known to exist with such a procedure. For example, it was well known CNBr linkage is subject to leaching Düring affinity chromatography, and that this varies with the agent used to remove the bound protein. See Goldenberg et al. Bioconjug Chem 1991 Jul-Aug;2(4):275-80 (Exhibit A); Lihme et al., J Chromatogr 1986 Apr 11;376:299-305 (Exhibit B). Düring, however, does not describe how protein was eluted from his Sepharose 4b absorbent. Düring translation, p. 88, end of first full paragraph ("After overnight incubation of the corresponding affinity gel at 4(C on the Denley roller, the bound protein is eluted in an Eppendorf flask under appropriate conditions and concentrated by ultrafiltration"). Furthermore, there is no indication in Düring that a control for column leaching was used or

even considered. In addition, as already discussed, Düring failed to adequately characterize the specificity of the AC38 antibody in the assay format for which it was used.

Accordingly, since there is no teaching or combination of teachings that could cure the deficiencies noted for Düring, and because the results obtained by the present inventors were clearly unexpected and surprising, especially in light of the lack of success of Düring, the obviousness rejection under §103 (a) fails as a matter of law. The examiner, therefore, is respectfully urged to withdraw the rejection of the claims.

REJECTION UNDER 35 U.S.C. § 103 OVER GOODMAN

The rejection of claims 21-40 and 42-68 under 35 U.S.C. § 103(a) as being allegedly obvious over Goodman is respectfully traversed. Claims 21-40, 42, 44-47, 49, 51-57, 64, and 68 have been cancelled herein, rendering the rejection moot as to these claims.

As already discussed under anticipation, Goodman's teachings at best are limited to expressing gamma interferon, a lymphokine that is a single polypeptide and is functionally distinct from immunoglobulin. With only a single sentence that mentions the production of immunoglobulins, the Goodman disclosure is not enabling for immunoglobulin. Goodman's passing reference to immunoglobulin expression is mere speculation, amounting to nothing more than an invitation to experiment. The Goodman reference fails to teach each and every limitation of the claimed invention.

Furthermore, as already mentioned, gamma interferon is structurally and functionally distinct from immunoglobulin light or heavy chains, the latter of which are immunoglobulin superfamily members. Also, it was known by the late 1980s that antibodies encompassing a heavy and a light chain are secreted through a complex interaction between the chains and other proteins. For example, heavy chain production in B cells was known to precede that of light chain production in ontogeny, and that the heavy chain binds to the BiP protein in the endoplasmic reticulum before heavy chain assembles with light chain. See e.g., Hass et al., Proc. Natl. Acad. Sci. USA 81:7185-7188 (1984) (copy attached as Exhibit C, p.7187, right column, citing reference 12, Burrous et al.. PNAS USA 78:564 (1981)). It

was also known that heavy chain production in the absence of light chain production was often fatal in mature B lymphocytes. See e.g., *Id.* (Exhibit C; p.7185, left column). Furthermore, if heavy chain were produced without light chains, the heavy chains were not secreted (see Pepe et al., J. Immunol. 137:2367-2372 (1986); copy attached as Exhibit D, p.2367, left column); The opposite was true, however, when light chains were secreted without heavy chains.

It is respectfully submitted, therefore, that the above noted deficiencies in the teachings of Goodman clearly demonstrate that no substantive foundation exists upon which to find the claims obvious over this reference. It is also submitted that no such teachings or combination of teachings exist that could cure the deficiencies noted for During. Moreover, as Applicants argued in the previous response, the unexpected results obtained from the instant invention was so surprising that the present inventor's work was featured on the cover of the prestigious journal Nature (Nov. 2: 342 (6245):76-78, 1989).

CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is urged to contact the undersigned by telephone to address any outstanding issues standing in the way of an allowance.

Respectfully submitted,

Date: January 28, 2002

By _____

Barry S. Wilson
Attorney for Applicant
Registration No. 39,431

FOLEY & LARDNER
P. O. Box 80278
San Diego, California 92138-0278
Telephone: (858) 847-6722
Facsimile: (858) 792-6773

Atty. Dkt. No.:EPI3007D
(formerly TSRI 184.2CON-3)

VERSION WITH MARKINGS TO SHOW CHANGES MADE

43. (Amended) A plant cell that contains [a nucleotide sequence that encodes] nucleotide sequences encoding a biologically functional multimeric protein not normally produced by the plant cell, wherein said multimeric protein is an immunoglobulin molecule, said nucleotide sequences encoding an immunoglobulin heavy and light chain polypeptide wherein each polypeptide contains a leader sequence forming a secretion signal; and immunoglobulin molecules encoded by said nucleotide sequences, wherein each leader sequence forms a secretion signal that is cleaved from said immunoglobulin heavy chain and light chain polypeptide following proteolytic processing.

48. (Amended) The plant cell of claim 43 wherein the [multimeric protein] immunoglobulin molecule forms a binding specific for a predetermined antigen.

50. (Amended) The plant cell of claim 43 wherein the [multimeric protein] immunoglobulin molecule is an abzyme.

58. (Amended) The plant cell of claim 43 wherein the immunoglobulin [product] molecule comprises an antibody.

59. (Amended) The plant cell of claim 43 wherein the immunoglobulin [product] molecule comprises a paratope.

60. (Amended) The plant cell of claim 43 wherein the [multimeric protein comprises a glycosylated] immunoglobulin molecule is glycosylated, said glycosylation being free of sialic acid.

65. (Amended) The [method] plant cell of claim 43 wherein the leader sequence is a [non-native] non-immunoglobulin leader sequence.

66. (Amended) The [method] plant cell of claim [65] 43, wherein the leader sequence is a yeast leader sequence.

67. (Amended) The [method] plant cell of claim [65] 43, wherein the leader sequenc is a plant leader sequence.

1: Bioconjug Chem 1991 Jul-Aug;2(4):275-80 Related Articles, NEW E

Specific interchain cross-linking of antibodies us bismaleimides. Repression of ligand leakage in immunoaffinity chromatography.

Goldberg M, Knudsen KL, Platt D, Kohen F, Bayer EA, Wilchek M.

Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel.

The extensive use of antibody-containing affinity columns in purification of biologically active compounds (e.g., genetically engineered proteins) is severely hampered by the leaching of antibody (or portions thereof) from the immunoaffinity resin during elution of the target antigen. One of the major proble this context is the combined use of reducing (i.e., thiols) and chaotropic (e.g., detergents and denaturants) agents in the elution step, which causes the disassociation of heavy and/or chains from the immobilized antibody, thereby contaminating resultant product. In order to overcome this problem, we hav cross-linked the four antibody chains at their sites of disulfide interlinkage, thus producing a single antibody chain. To accomplish this, interchain disulfide bonds were reduced, and resultant thiol groups were cross-linked by using bifunctional specific reagents (particularly bismaleimides). Cross-linking c to 95% of the available SH groups produced was achieved wi concomitant retention of antigen-binding activity. The cross-linked antibody was immobilized onto CNBr-activated Sephar and the resultant column was found to be substantially more stable to harsh elution conditions than similar columns which contain the un-cross-linked antibody.

PMID: 1772910 [PubMed - indexed for MEDLINE]

EXHIBIT A

1: J Chromatogr 1986 Apr 11;376:299-305 Related Articles, NEW

Divinylsulphone-activated agarose. Formation of stable and non-leaking affinity matrices by immobilization of immunoglobulins and other proteins.

Lihme A, Schafer-Nielsen C, Larsen KP, Muller KG, Bog-Hansen TC.

Divinylsulphone-activated agarose is an attractive alternative several of the activated supports usually used. Unlike CNBr-activated gels, it does not leak the immobilized protein at hig pH. It reacts readily with proteins at near-neutral pH (unlike epoxy-activated supports). Generally, divinylsulphone-activa agarose reacts with amino, hydroxyl, and sulphydryl groups, allowing immobilization of a wide spectrum of ligands. Moreo it is available in an aqueous suspension free of organic solve and neither requires time-consuming swelling nor washing.

PMID: 3519637 [PubMed - indexed for MEDLINE]

EXHIBIT B

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 7185–7188, November 1984
Immunology

Immunoglobulin heavy chain toxicity in plasma cells is neutralized by fusion to pre-B cells

(immunoglobulin chain loss/isoelectric focusing of heavy chain)

INGRID G. HAAS* AND MATTHIAS R. WABL*†

*Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstrasse 37-39, D-7400 Tübingen, Federal Republic of Germany; and †Basel Institute for Immunology, Grenzacherstrasse 487, CH-4058 Basel, Switzerland

Communicated by Niels K. Jerne, July 9, 1984

ABSTRACT A plasma cell hybridoma frequently loses its immunoglobulin heavy (H) chain spontaneously but rarely is production of its light (L) chain lost. Upon fusion to a pre-B-cell hybridoma that produces no Ig chain, the L chain is frequently lost. In cells without the L chain the H chain, which is derived from the plasma cell, is not chemically modified. Our results indicate that, in pre-B cells, but not in plasma cells, there must be a mechanism that neutralizes the toxic effect of free H chain.

Myelomas and hybridomas derived from plasma cells secrete immunoglobulin consisting of heavy (H) and light (L) chains. From such cell lines, subclones that have lost H chain expression can be recovered, but they still secrete the L chain (1, 2). The L chain can then be lost at the same frequency as the H chain. On the other hand, it seems to be very difficult to recover cells that have lost L chain expression but that still synthesize the H chain (1–3) except when the cells are mutagenized (4) or express a mutant H chain (5–7). These observations have led to the view that the free H chain is toxic to the cells (2, 3). However, cells of an earlier differentiation stage, pre-B cells, synthesize intracellular H chain in the absence of the L chain (8). To explain this difference, several possibilities have been proposed: (*i*) the rate of H chain synthesis in pre-B cells is too low to damage the cell (9); (*ii*) the pre-B cell synthesizes a different H chain that is not toxic to the cell (2); or (*iii*) in pre-B cells, there is a special protein that neutralizes the toxic effect of the free H chain (10, 11).

Here we report that hybridomas derived from pre-B cells are not different from plasma cell hybridomas with respect to their rate of H chain synthesis and steady-state level of H chain. However, they do not synthesize the L chain. In consequence, we wanted to answer the question whether there are pre-B-cell hybridomas that can synthesize a free H chain, which has been shown to be toxic in plasma cell hybridomas.

MATERIALS AND METHODS

Cell Lines. Sp2 and GK14.1 were established and provided by G. Köhler (Basel). The cell lines are derived from fusions between spleen cells and a myeloma, X63 Ag8, and synthesize IgG2b. Sp2.0 is an azoguanine-resistant subclone of Sp2 and has lost Ig expression. Cell lines H32-21, H32-3, H32-8, and H6 are derived from fusions between γ2b-synthesizing subclones of the Abelson virus-transformed pre-B-cell line 18-81 and X63 Ag8653 (12). Clone H62 is a subclone of H6 and synthesizes no Ig chain. NORA hybridomas were made by fusion of Sp2 HL Ag14 and H62. SPSP hybridomas are derived from a fusion between Sp2.0 and Sp2. Cell fusion was carried out as described (12). The genealogy of the various hybridomas is given in Fig. 1.

Isolation of Subclones with Ig Chain Loss. Soft agar cloning and antiserum overlay was carried out according to the method of Coffino and Scharff (1). When an antiserum against IgG2b (γ2b,κ) was used, about 1% of the cells formed colonies without precipitation. These clones were isolated, and their Ig expression was analyzed by immunofluorescence. Clones of interest were grown in mass culture and further analyzed by immunoprecipitation and electrophoresis.

NaDodSO$_4$/Polyacrylamide Electrophoresis. Cell labeling, immunoprecipitation, and NaDodSO$_4$/polyacrylamide gel electrophoresis were carried out as described (12). Pulse labeling (30 min) was carried out by the addition of [^{35}S]methionine to cells that had been incubated in methionine-free select medium/10% dialyzed fetal calf serum for 1 hr. The amount of [^{35}S]methionine incorporated into the precipitated proteins was measured after gel fractionation by scintillation assay of the solubilized gel slices.

Isoelectric Focusing. [^{35}S]Methionine-labeled Ig precipitates were dissolved in 9.5 M urea/2% Nonidet P-40/2% Ampholine (pH 5–11)/5% 2-mercaptoethanol and applied to isoelectrofocusing slab gels. The gel composition was according to O'Farrell (13). Electrophoresis was carried out for 1 hr at 250 V, for 12 hr at 400 V, and finally for 1 hr at 800 V. The proteins were visualized by fluorography.

Immunofluorescence. The purification and fluorochrome conjugation of goat antibodies specific for mouse H chain isotypes and the methods for immunofluorescence detection of intracellular Ig have been described (14).

RESULTS

The Rate of Ig H Chain Synthesis and the Steady-State Level of the H Chain in Plasma Cell- and in Pre-B Cell-Derived Hybridomas Are of the Same Order of Magnitude. The pre-B cell is characterized by the synthesis of intracellular H chain in the absence of the L chain. The free H chain is toxic in plasma cells, but pre-B cells may survive because they synthesize the H chain in small amounts (15, 16) that would not damage the cell. To test this hypothesis, we increased the amount of free H chain present in pre-B cells by fusion to a myeloma. The steady-state level of RNA specific for the H chain is the same in pre-B-cell hybridomas and in plasma cell hybridomas (16).

By measuring the short-term incorporation of radiolabeled methionine into the H chain (Fig. 2), we have compared the rate of Ig H chain synthesis in pre-B-cell-derived hybridomas with that of plasma cell-derived hybridomas. The amount of radioactivity incorporated into the various H chains was of the same order of magnitude. Also, the steady-state level of the H chain, as determined by long-term label-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: H chain and L chain, heavy and light chain, respectively, of Ig; BiP, H chain binding protein.

EXHIBIT C

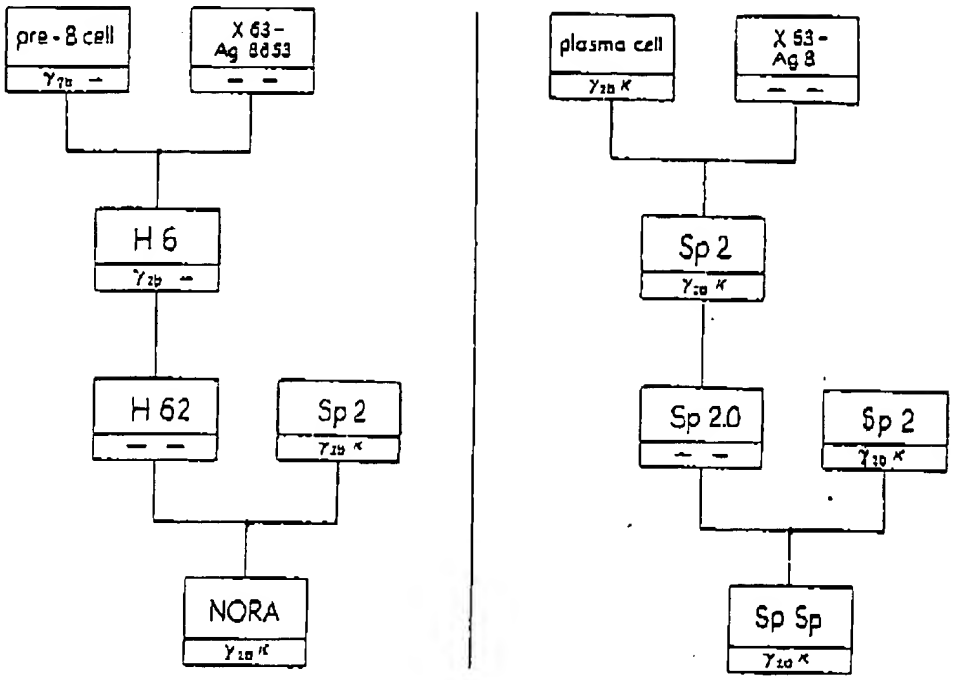

FIG. 1. Genealogy of selected hybridomas derived from a pre-B cell, or from a plasma cell, or from both. The kind of Ig chain synthesized is given below the clone designation.

ing (Fig. 3) and by immunofluorescence intensity (data not shown), was not different. This does not formally exclude that pre-B cells survive the expression of free H chain because of a low rate of synthesis, but there would still remain the question of why the H chain is not toxic for pre-B-cell-derived hybridomas as it is for plasma cell-derived hybridomas.

A Given H Chain Is Toxic in Plasma Cells but Not in Pre-B Cells. Because pre-B-cell-derived hybridomas can survive high levels of the H chain, we wanted to know whether they are able to survive the expression of a H chain, the toxicity of which has been demonstrated in plasma cells. For that purpose, we fused a plasma cell hybridoma to a pre-B-cell hybridoma that had lost its own H chain expression.

The Sp2 cell line is derived from fusion of a plasma cell with a myeloma (Fig. 1). It synthesizes both H and L chain and exhibits the H chain toxicity phenomenon (2). We confirmed this by recovering Ig chain-loss-variants according to the method of Coffino and Scharff (1). Of 74 subclones, 69 expressed no H chain, one expressed no L chain (that is, expressed H chain only), and 4 did not express any Ig chain at all (Table 1). We then fused the Sp2 cell line to a pre-B-cell-derived hybridoma, H62, that had lost the expression of its own Ig chain (Fig. 1). The resulting hybrid cell line NORA 4 secreted H and L chain from the Sp2 parent line (Figs. 3 and 4). The NORA cell line tolerates the H chain in the ab-

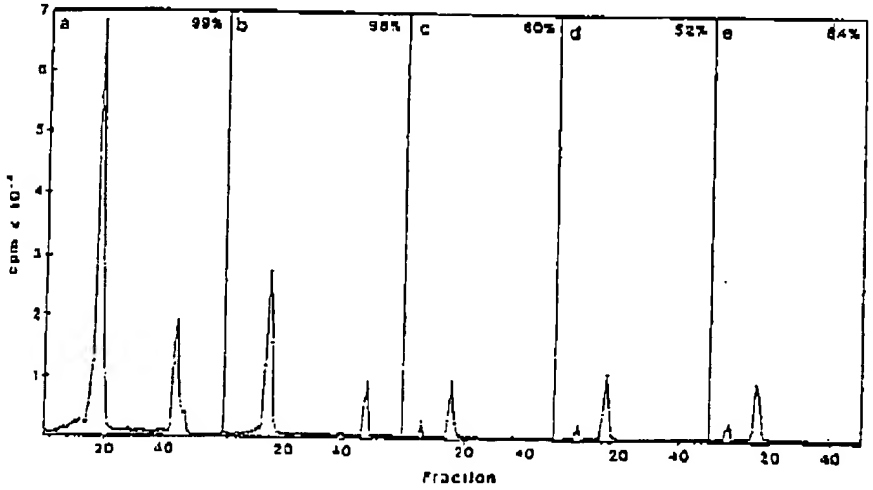

FIG. 2. Amount of radioactivity incorporated into immunoprecipitated proteins after separation on polyacrylamide gel. GK1a.1 (*a*) and Sp2 (*b*) are plasma cell-derived hybridomas synthesizing both H and L chain; H32-21 (*c*), H32-3 (*d*), and H32-8 (*e*) are pre-B-cell-derived hybridomas synthesizing a H chain that is associated with BiP (11). The indicated percentages of cells synthesizing Ig were determined by immunofluorescence.

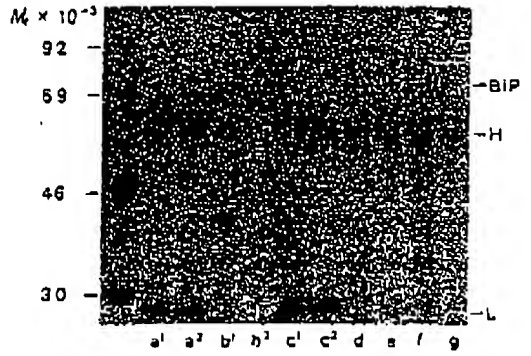

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis analysis of the Igs of various hybridomas synthesizing both H and L chain or synthesizing the H chain only. Sp2 (lanes a) and NORA 4 (lanes c) secrete both H and L chain; SPSP 1.55 (lanes b), NORA 4.2 (lane d), NORA 4.16 (lane e), NORA 4.8.20 (lane f), and the pre-B-cell hybridoma H 61 (lane g) synthesize the H chain without the L chain but do not secrete it.

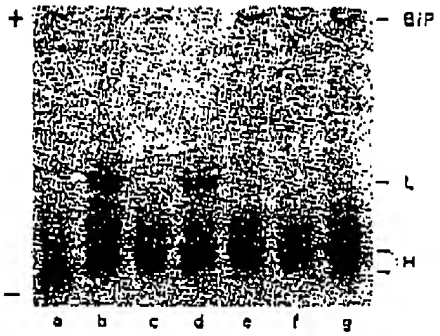

FIG. 4. Isoelectric focusing analysis of intracellular Ig of various hybridomas synthesizing both H and L chain or synthesizing H chain only. Lanes: a, pre-B-cell hybridoma H 61 (γ2b); b, Sp2 (γ2b,κ); c, SPSP 1.55 (γ2b); d, NORA 4 (γ2b,κ); e, NORA 4.2 (γ2b); f, NORA 4.16 (γ2b); g, NORA 4.8.20 (γ2b). The γ2b chain of H 61 has a variable region different from the one of the Sp2 cells and their hybridomas.

sence of the L chain. Ten out of 55 subclones of NORA 4 with Ig chain loss synthesized Ig H chain without the L chain, 42 synthesized the L chain only, and 3 did not synthesize any Ig chain at all (Table 1). A subclone of NORA 4, NORA 4.8, that synthesizes both H and L chain, showed the same chain loss distribution (Table 1). In no instance was the H chain secreted. As a control to account for the increased chromosome numbers of the NORA 4 hybrid as compared with Sp2, we fused the Sp2 cell line to a nonproducing subclone, Sp2.0, generating SPSP hybridomas (Fig. 1). From these hybridomas, subclones synthesizing free H chain should be rare. Indeed, of 125 subclones with Ig chain loss, we recovered only 1 subclone expressing free H chain (Table 1). Of these subclones, 122 had lost the H chain and two had lost both Ig chains. Comparison of the H chain of hybridomas synthesizing both H and L chain or the H chain alone revealed no difference in size (Fig. 3) nor in isoelectric focusing behavior (Fig. 4). Since in this case, the H chain is one that is known to be toxic, we conclude that in pre-B cells there is a mechanism neutralizing the toxic effect of free H chains.

DISCUSSION

Why are free Ig H chains toxic in plasma cells but not in pre-B cells? From the plasmacytoma MOPC 21, a variant could be recovered that synthesizes only the H chain. The mutant H chain of this variant lacks the last 67 carboxyl-terminal amino acids and forms polymers of at least 20 H chains (9). This polymerization is probably due to free sulfhydryl (SH) groups on the H chain that would normally form the L-H bridge. The normal H chain probably could also polymerize in the absence of L chains but would form much larger insoluble complexes that would damage the cell. Polymerization of L chains cannot occur because no additional free SH groups are available once the L chain has formed a dimer. It is of interest that H chains are predominantly found as monomers or dimers in pre-B cells (11), as well as in the H chain-synthesizing subclones of the NORA 4 hybrid line (data not shown). Thus, neutralization of the toxic effect of the free H chain may be achieved by the prevention of H chain polymerization—for example, by an enzyme that alters the reactive SH groups of the Ig H chain. Since we did not find any difference in the isoelectric focusing pattern of the intracellular H chains of Sp2, NORA 4, and of those subclones synthesizing no L chain (Fig. 4), one can postulate a protein that either rapidly degrades accumulating free H chain or protects the reactive SH groups without chemical modification. We have already described a protein (heavy chain-binding protein, BiP) that binds to Ig H chains not associated with the L chain (11). In all NORA subclones that have lost the L chain (some of them are shown in Fig. 3), the H chain is associated with the BiP. If the BiP is neutralizing H chain toxicity it should be less active in plasma cells than in pre-B cells.

Whatever the mechanism for neutralization of H chain toxicity, our results demand an explanation of why H chain synthesis precedes L chain synthesis in B-cell ontogeny (10).

Table 1. Ig chain loss pattern in various hybridomas

Hybridoma	No. of subclones		
	H chain lost	L chain lost	H + L chain lost
Sp2	57	0	4
Sp2.68	12	1	0
Total	69	1	4
NORA 4	42	10	3
NORA 4.8	42	12	0
Total	84	22	3
SPSP 1	59	1	2
SPSP 2	63	0	0
Total	122	1	2

We thank Drs. J. Johnson (Munich) and C. Steinberg (Basel) for discussions.

1. Coffino, P. & Scharff, M. D. (1971) *Proc. Natl. Acad. Sci. USA* 68, 219–223.
2. Köhler, G. (1980) *Proc. Natl. Acad. Sci. USA* 77, 2197–2199.
3. Wilde, C. D. & Milstein, C. (1980) *Eur. J. Immunol.* 10, 462–467.
4. Morrison, S. L. & Scharff, M. D. (1975) *J. Immunol.* 114, 655–659.
5. Morrison, S. L. (1978) *Eur. J. Immunol.* 8, 194–199.
6. Sonenshein, G. E., Siekevitz, M., Siebert, G. R. & Gefter, M. L. (1978) *J. Exp. Med.* 148, 301–312.
7. Cowan, N. J., Secher, D. S. & Milstein, C. (1974) *J. Mol. Biol.* 90, 691–701.
8. Burrows, P. D., Lejeune, M. & Kearney, J. F. (1979) *Nature (London)* 280, 838–841.
9. Argon, Y., Burrone, O. R. & Milstein, C. (1983) *Eur. J. Immunol.* 13, 301–305.

10. Wabl, M. & Steinberg, C. (1982) *Proc. Natl. Acad. Sci. USA* 79, 6976–6978.
11. Haas, I. G. & Wabl, M. (1983) *Nature (London)* 306, 387–389.
12. Burrows, P. D., Beck, G. B. & Wabl, M. R. (1981) *Proc. Natl. Acad. Sci. USA* 78, 564–568.
13. O'Farrell, P. H. (1975) *J. Biol. Chem.* 250, 4007–4021.
14. Kearney, J. F. & Lawton, A. R. (1975) *J. Immunol.* 115, 671–676.
15. Raff, M. D., Megson, M., Owen, J. J. T. & Cooper, M. D. (1976) *Nature (London)* 259, 224–226.
16. Wabl, M. R. & Burrows, P. D. (1984) *Proc. Natl. Acad. Sci. USA* 81, 2452–2455.

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

0022-1767/86/1377-2367$02.00/0
The Journal of Immunology

Vol. 137, 2367–2372, No. 7, October 1, 1986
Printed in U.S.A.

GENE TRANSFER OF IMMUNOGLOBIN LIGHT CHAIN RESTORES HEAVY CHAIN SECRETION[1]

VINCENT H. PEPE,* GAIL E. SONENSHEIN,* MARK I. YOSHIMURA,† AND MARC J. SHULMAN[2†]

*From the *Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118; and the †Rheumatic Disease Unit, Wellesley Hospital and the Department of Medical Biophysics, University of Toronto, Toronto, Ontario M4Y1J3, Canada*

Several lines of evidence suggest that immunoglobulin (Ig) light (L) chain plays a role in the secretion of heavy (H) chain. For example, myeloma variant lines, which synthesize the Ig H chain but not the L chain, fail to secrete H chain protein. Here we have tested directly the role of chain assembly in the control of Ig secretion by the transfer of functional L chain genes into two such L chain-defective myeloma mutants. A λ2 or κ L chain gene was introduced into variant lines of the mouse myelomas MOPC 315 (IgA, λ2) or PC7 (IgM, κ), respectively. Although the two mutant lines are unable to secrete the H chain they produce, rescue of secretion of complete Ig protein molecules (IgA or IgM) was observed after transfection. These results imply that the secretory apparatus of these cells is intact and that the failure to secrete free H chain reflects a structural feature intrinsic to that protein. The implications of these results with respect to control of secretion of multi-subunit proteins are discussed.

The immunoglobulin (Ig) heavy (H) and light (L) chains are assembled and processed intracellularly and then secreted together as a mature, functional protein (1). A normal L chain can usually be secreted in the absence of H chain. For example, Bence-Jones proteins are derived from human myelomas that secrete free L chain (2), whereas in the mouse system, there exist mutant myeloma and hybridoma cell lines that do not produce H chain but continue to secrete L chain (3). The situation for the normal H chain is different from that of the L chain. Mutant cell lines have been described that synthesize a full length H chain but no L chain (3, 4). These H chains are not secreted. Construction of hybrid lines between H chain and L chain variant lines results in H chain secretion in the form of assembled Ig molecules (5, 6). Although these results suggest that the normal full-length H chain can be secreted only when assembled with the L chain, other explanations are possible. For example, the L chain-nonproducing cell lines might have undergone a second, unrelated mutation that reduces their capacity to secrete Ig. We show here that the first hypothesis is correct, in that for two cell lines that produce H chain only, the transfer of a functional L chain gene restores H chain secretion.

MATERIALS AND METHODS

MOPC 315 cell growth conditions and measurement of Ig polypeptide synthesis and secretion. MOPC 315 cells were grown and maintained by serial passage in Dulbecco's modified Eagle's medium (DME) supplemented with 16% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 mM nonessential amino acids, 4 mM glutamine, and 40 mM glucose.

To assess intracellular Ig L and H chain synthesis, cultures were pulse labeled for 20 min with a mixture of six radiolabeled amino acids: 20 μCi/ml of [^{3}H]leucine, [^{3}H]lysine, [^{3}H]proline, [^{3}H]phenylalanine, [^{3}H]tyrosine, and 20 μCi/ml of [^{35}S]methionine. Washed cells were resuspended in 50 mM Tris-HCl, 50 mM NaCl and were lysed by the addition of Triton X-100 and sodium deoxycholate to 1% final concentration each, as described (6). After removal of nuclei by centrifugation at 2000 rpm for 10 min, samples of cell cytoplasmic lysates were removed for determination of trichloroacetic acid-insoluble radioactivity. Equal aliquots of cell lysates were immunoprecipitated with rabbit anti-mouse IgA protein and a goat anti-rabbit Ig antisera (Miles Yeda). Precipitates were dissolved in 50 mM Tris, pH 6.8, 2% SDS, where noted proteins were reduced with mercaptoethanol (0.5%). Samples were analyzed by SDS-PAGE as described (8 to 13%) (7).

To assess secretion of Ig protein, cultures were labeled for a 4-hr period with the amino acid mixture described above, and samples of the media immunoprecipitated and analyzed by SDS-PAGE as above.

PC7-derived cell lines and measurement of Ig polypeptide synthesis and secretion. The cell line PC7 secretes IgM specific for the hapten phosphorylcholine (PC)[3] (4). From this cell line, we isolated the mutant 574, which lacks the gene for PC-specific L chain (M. Baker, personal communication). Mutant 574 produces, but does not secrete, an apparently normal μ chain. The mutant cell line 400 lacks the μ gene of the PC7 parent line (unpublished results); it continues to synthesize the κ L chain.

PC7 cells were incubated in [^{14}C]leucine to label intracellular and secreted Ig. The μ and κ chains were precipitated by reacting with a mixture of rabbit anti-μ and anti-κ sera (Daymar Laboratories). Non-reduced samples were resolved on 4% polyacrylamide gels containing 0.1 M Tris-bicine pH 8.3, 0.1% SDS, and 0.7% *N,N'*-diallyltartardiamide as described (8). Free μ and κ chains, obtained after reduction, were analyzed with the gel system described by Laemmli (9).

Construction of vectors bearing λ2 and κ L chain genes. The 6.6-kb Eco RI genomic fragment containing the λ2 MOPC 315 L chain gene (10) was inserted into the Eco RI site of the pSV2-gpt[3] vector containing the SV40 enhancer (11), as shown in Figure 1a. The resulting vector, pCgt-λ2, kindly provided by H. Murialdo (University of Toronto), was transfected into *E. coli* strain HB101.

The 7-kb S107 fragment (12), encoding the κPc L chain, was inserted into the Bam HI site of the plasmid pSV2-neo (13). The recombinant plasmid, pNeo-κPc, containing the insert in the orientation shown in Figure 1b, was transfected into *E. coli* strain K803.

Protoplast fusion. Fusion of bacterial protoplasts with myeloma

Received for publication November 30, 1985.
Accepted for publication June 26, 1986.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by grants to M. J. S. from the Allstate Foundation, The Arthritis Society (Canada), The Medical Research Council (Canada), and The Wellesley Hospital Research Institute, and to G. E. S. from the National Institutes of Health (AI 16051 and CA 38395). V. H. P. has been supported by National Institutes of Health Training Grant T32 HL 07052.
[2] Current address: Department of Immunology, Medical Sciences Building, University of Toronto, Toronto, Ontario M5S1A8, Canada.

[3] Abbreviations used in this paper: PC, phosphorylcholine; HAT, hypoxanthine-aminopterin-thymidine; gpt, guanine phosphoribosyl transferase.

EXHIBIT D

cells was performed as described (14, 15). A suspension of bacterial cells transformed with the plasmid, which had been amplified overnight in the presence of chloramphenicol, was centrifuged at 3000 rpm for 30 min, washed once with 0.9% NaCl, and resuspended in 0.05 M Tris, pH 8, 20% sucrose at a final concentration of 1×10^{10} cells/ml. To 1 ml of bacterial suspension, 0.2 ml lysozyme (1 mg/ml in 0.25 M Tris, pH 8.0) was added, and the mixture was incubated for 5 min on ice. After subsequent addition of 0.4 ml of 0.25 M EDTA, pH 8.0, the mixture was again incubated for 5 min on ice, then diluted with 0.4 ml 0.05 M Tris, pH 8.0, and incubated at 37°C for 30 min with occasional mixing. The suspension was diluted with 10 ml of medium containing 10% sucrose and 0.0125 M $MgCl_2$; 0.1 ml of 1 mg/ml DNase was added, and the mixture was incubated at room temperature for 15 min. The protoplasts (1×10^9 cells) were pelleted, supernatant was removed, and 10^7 myeloma cells were layered on top by centrifugation. The protoplast and cell mixture was gently resuspended in 1 ml PEG fusion buffer (0.85 gm PEG 1000, 1 ml DME, 0.2 ml dimethyl sulfoxide). After a 15-sec exposure, the cell suspension was diluted with DMEM without serum, and cells were pelleted by centrifugation, resuspended in medium, and distributed in microtiter wells. For the transfer of the λ2 gene, cells were placed after 3 days in HAT (hypoxanthine-aminopterin-thymidine) selective medium and then cloned in soft agar. For the transfer of the spe gene, cells were plated at 10^4 and 10^5 cells/ml in multiwell dishes; after 2 days of incubation, the medium was supplemented with 1 mg/ml of the antibiotic G418 [13].

RESULTS

Synthesis and secretion of IgA protein by MOPC 315 LV-1 variant after λ2 L chain gene transfer. LV-1 was originally isolated as a nonsecreting variant of the mouse myeloma MOPC 315 (IgA, λ2) tumor (16). It was adapted to growth in tissue culture and was subsequently demonstrated to have lost the ability to synthesize the λ2 L chain (6). LV-1 synthesizes an altered α H chain, approximately 3000 daltons smaller than the wild-type chain. This altered H chain is not secreted, but instead is degraded intracellularly with a half-life of approximately 30 min (6). To facilitate isolation of transfectant colonies of LV-1 cells, a λ2 L chain gene cloned in the vector pSV2-gpt and an LV-1 derivative defective in hypoxanthine phosphoribosyl transferase (HPRT) activity were employed (Fig. 1*a*). The pSV2-gpt vector bears the bacterial gene for guanine phosphoribosyl transferase (gpt) and confers on HPRT⁻ cells the capacity to grow in HAT-containing medium (11). The cloned DNA was transferred by protoplast fusion to the LV-1 derivative line defective in HPRT activity, as described in *Materials and Methods*. Five HAT-resistant transformants were isolated and recloned for study. The individual lines were tested for λ2 L chain synthesis, and the levels were compared with the parental LV-1 line, as negative control, and with a hybrid line, HL-2, derived by fusion of LV-1 with V-1, a λ2 L chain-producing variant of MOPC 315, described previously (17). The results are displayed in Fig. 2*A*. The profile seen with HL-2 represents approximately wild-type amounts of λ2 and α chain synthesis for the MOPC 315 myeloma line. (The additional bands of immunoprecipitated material are the translation products of aberrant α H chain RNA species transcribed from the excluded α H chain constant region allele.) (17, 18) The level of λ2 chain production by the transformants varied, but was always much lower than wild-type cells. (Values between 10- and 50-fold lower were obtained, data not shown.) The pattern from the transformant that produces the highest level of λ2 chain TLH-29 is shown in Figure 2*A*. As expected, no synthesis of L chain was detectable with LV-1, as observed previously (6).

The ability of the TLH-29-transfected cell line to secrete Ig was analyzed (Fig. 2*B*). Both α chain and λ2 chain are secreted by TLH-29, whereas no secreted Ig is observed in the media of the parental line LV-1, as mentioned above and reported previously (6). The level of secreted IgA by TLH-29 is significantly lower than that from HL-2 and is commensurate with the lower level of λ2 chain produced intracellularly by the transfected line. The band visible above the λ2 chain in the TLH-29 and HL-2 lanes has been identified as the J chain by specific immunoprecipitation (7). This protein is involved in the formation of multimeric forms of IgA protein (19) and is only secreted attached to α chain (20, 21). The other transfected lines also secreted IgA protein, although again the levels were lower commensurate with the amount of λ2 chain production (data not shown).

Although the λ2 chain of IgA is not disulfide bonded to the H chain and migrates separately in an SDS gel system, the formation of H chain multimers containing more

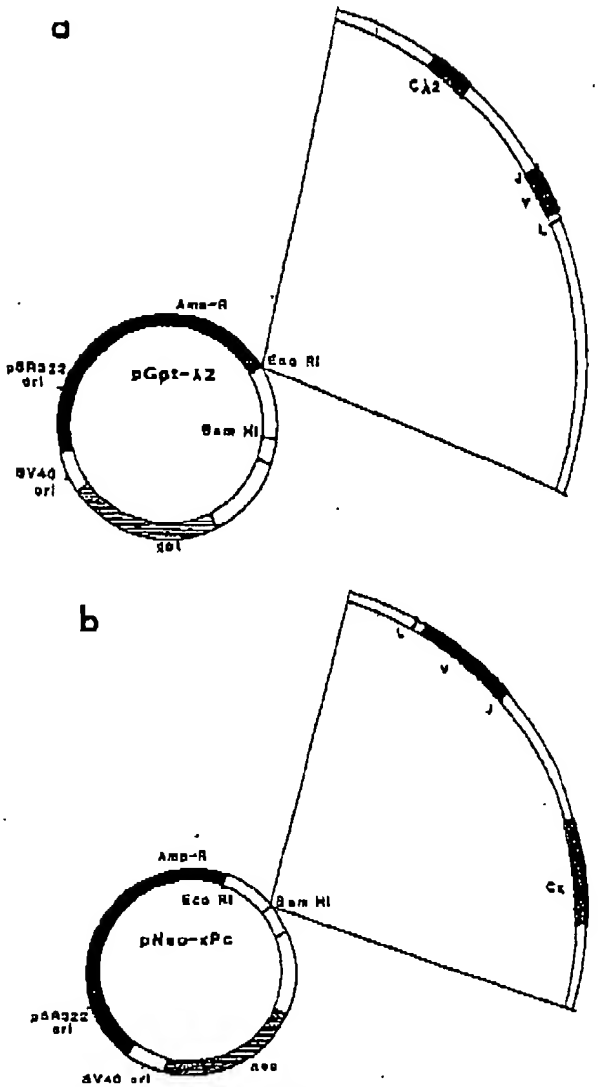

Figure 1. Construction of vectors bearing λ2 and κ L chain genes. *a*. The 6.6-kb cloned MOPC 315 L chain gene (10) was inserted into the Eco RI site of the pSV2-gpt vector. *b*. The 7-kb S107 fragment (12), encoding for the κPc L chain, was inserted into the Bam HI site of the plasmid pSV2-neo.

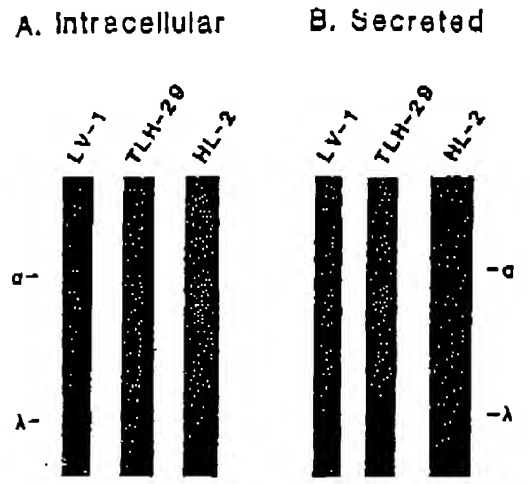

Figure 2. Rescue of Ig H chain secretion after transfer of λ2 gene. Bacterial protoplasts of strain HB101 bearing the plasmid pCpt-λ2 (Fig. 1a) were fused with LV-1. After 3 days, cells expressing the gpt gene were selected in HAT medium and cloned in soft agar. *A.* Analysis of intracellular Ig L and H chain synthesis. Cultures were pulse labeled for 20 min with a mixture of six radiolabeled amino acids: 20 μCi/ml of [^{3}H]leucine, [^{3}H]lysine, [^{3}H]proline, [^{3}H]phenylalanine, and [^{3}H]tyrosine and 20 μCi/ml of [^{35}S]methionine. Cell lysates were immunoprecipitated with rabbit anti-mouse IgA protein and a goat anti-rabbit Ig antiserum. Precipitates were dissolved in 50 mM Tris, pH 6.8, 2% SDS, proteins were reduced with mercaptoethanol (0.5%), and equal samples were analyzed by SDS-PAGE (8 to 13%). *B.* Analysis of secreted Ig proteins. Cultures were incubated in the presence of the radiolabeled amino acid mixture for a 4-hr period; fractions of the media were immunoprecipitated and analyzed by SDS-PAGE as above.

Figure 3. Structure of secreted IgA molecules after transfer of λ2 gene. Secreted IgA protein was radiolabeled and immunoprecipitated as described in Figure 2. Precipitates were dissolved in 50 mM Tris, pH 6.8, 2% SDS, and samples were analyzed by SDS-PAGE (8 to 13%) without reduction.

than two H chain molecules is diagnostic of assembled IgA (21). The mobility of the secreted Ig protein under conditions where disulfide bonds were not reduced indicated the formation of dimers and tetramers of H chain, consistent with normal multimeric IgA formation (Fig. 3). Thus, although suggestive, SDS-PAGE cannot be used to prove the formation of complete multimeric IgA forms even though, as seen below, pentameric IgM is secreted from μ chain transformants.

The kinetics of Ig protein secretion from TLH-29 were measured by pulse-chase analysis. The time course of appearance of IgA into the medium is shown in Figure 4.

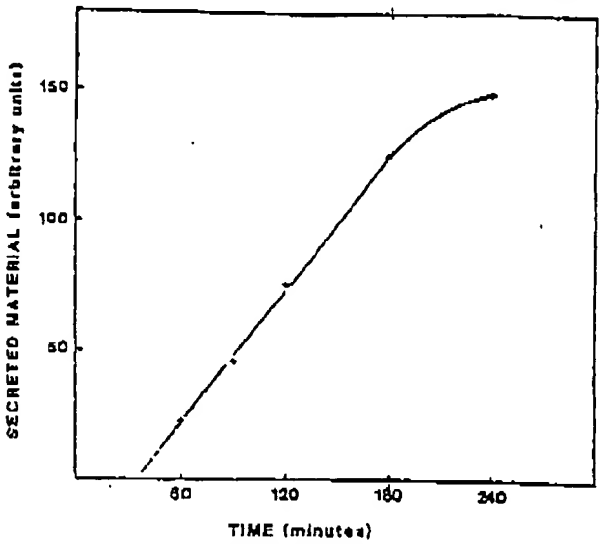

Figure 4. Kinetics of H chain secretion from LV-1 after transfer of the λ2 L chain gene. Cultures of TLH-29 were pulse labeled for 20 min as above, then washed and resuspended in fresh, warm medium. After the indicated times of chase, samples were removed and the media were analyzed for secreted α chains, as indicated in Figure 2. The autoradiograms were quantitated by scanning densitometry and integration of peak areas.

By extrapolation, secretion of newly synthesized IgA occurs after approximately 30 to 40 min. Similar values have been obtained for wild-type MOPC 315 cells (22). Thus, the time for secretion of IgA in the transformant TLH-29 line is approximately normal. Furthermore, examination of the profiles of labeled intracellular proteins indicates that expression of λ2 light chain in LV-1 cells results in stabilization of the H chain protein from degradation. The altered H chain polypeptide synthesized in LV-1 cells normally turns over with a half-life of approximately 30 min (6), and thus cannot be detected after the 240-min chase period (Fig. 5). In contrast, the kinetics of clearance of intracellular IgA from TLH-29 are similar to those observed with wild-type MOPC 315 cells (6, 22) and with the hybrid HL-2 line (Fig. 5). Thus, stabilization of the H chain from the normally rapid turnover observed in LV-1 cells occurs after expression of the introduced L chain gene.

Rescue of IgM secretion after κ L chain gene transfer. The hybridoma cell line PC7 produces IgM(κ), which is specific for the hapten PC. Using a suicide selection method (4), we obtained a mutant, 574, which produces a μ H chain that it does not secrete. From the results of Gearhart et al. (23), the PC7 cell line is expected to express the same germ-line PC-specific variable genes as are expressed in the myeloma S107. Accordingly, the κ gene cloned from S107 (Fig. 1*b*) (12) was transferred into the mutant 574 cell line. This vector carries the *neo* resistance gene, allowing for selection with the antibiotic G418 (12).

Twenty G418-resistant colonies were initially screened, of which 13 (65%) were positive for PC hemolysis (performed as described in Table I). The PC-specific lysis activity in the culture medium of these transformants ranged from nil up to the normal level. Of these, two were chosen (T574/κpc-2 and T574/κpc-10), which showed approximately normal activity, and one (T574/

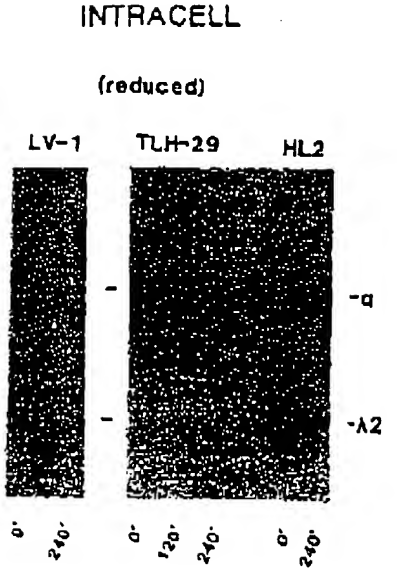

Figure 5. Pulse-chase analysis of intracellular IgA protein within LV-1 after transfer of the λ2 L chain gene. Cultures of TLH-29 were pulse labeled for 20 min as above, washed, and resuspended in fresh, warm medium. Samples, removed at the indicated times, were analyzed by immunoprecipitation and SDS-PAGE, as described in Figure 2, for L and H chains. Cultures of LV-1 and the hybrid line HL-2 were similarly treated; results for the 20- and 240-min time points are shown for comparison.

TABLE 1

Functional activity of IgM secreted by transformants[a]

Cell Lines	Hemolysis Titer	
	PC specific	μ specific
Wild type (PC7)	3^2	3^2
Mutant (574)	0	0
Mutant (400)	0	0
Transformants		
T574 (κpc-2)	3^2	3^2
T574 (κpc-10)	3^2	3^2
T574 (κpc-11)	0	0

[a] Culture supernatants were prepared by growing 10^6 viable cells/ml for 24 hr at 37°C. Supernatants of the cultures were threefold serially diluted, undiluted supernatant being the most concentrated material. Hemolysis titer was scored as the dilution at which a 2-μl sample caused hemolysis (4). IgM concentration was measured by the capacity of the culture supernatant to lyse protein A-coupled red cells in the presence of anti-μ serum (4, 24) (μ-specific hemolysis titer). PC-specific hemolysis was assayed directly on red cells coupled with PC-ONS ester (4).

κpc-11) with no activity (Table I). It should be noted that the precision of the hemolysis assay is about 1 dilution step (threefold).

The analysis of intracellular preparations (Fig. 6A) indicates that the parental and transformant cell lines synthesize the μ H chain. As expected, the transformants positive for PC-specific hemolysis synthesize the L chain; conversely, the transformant that was negative for PC-specific hemolysis showed no detectable intracellular κ L chain. The level of κ chain production is consistent with the hemolysis values within the accuracy of the assay. For comparison, results have been included for the PC7 mutant cell line 400, which lacks the μ gene but continues to synthesize the κ L chain at a very low level. Secreted IgM is analyzed in Figure 6B; the transformants that make κ chain also secrete μ H chain. The mobility of the material where disulfide bonds were not reduced indicates that the IgM is secreted in the pentameric form (Fig. 6C).

The low frequency of myeloma and hybridoma mutants making only H chain has suggested that high-level production of normal H chain in the absence of L chain might often be toxic (25). Our results to date suggest that the μ H chain of mutant 574 is normal. As shown in Figure 6, it is of normal size and can be incorporated into pentameric IgM. To assess its PC binding and complement activity capacity more quantitatively, we measured its hemolytic titer on PC-coupled red cells. The IgM concentration was measured by titering its capacity to induce lysis of protein A-coupled red cells in the presence of anti-μ serum (4) (Table I). These results indicate that the IgM made by L chain transformants of mutant 574 has normal activity. We are therefore unable to detect any functional (Table I) or any structural difference (Fig. 6) between these IgM molecules and conclude that the mutant 574 makes a normal μ H chain.

DISCUSSION

Assembly plays an important role in the secretion of Ig protein. Here we have demonstrated that the introduction of an appropriate λ or κ L chain gene restores secretion of α or μ H chain as assembled IgA or IgM molecules. These results directly demonstrate that L chain production is normally required for a cell to secrete H chain. This result explains the rescue of H chain secretion by myeloma H chain-producing cells after hybrid formation with L chain-producing cells, as well as the failure of investigators from several laboratories to isolate myeloma variants capable of secreting free normal H chain (5, 6, 25). This conclusion is also consistent with the observation of Valle et al. (26) that Ig H chain dimers accumulate in oocytes microinjected with myeloma H chain mRNA and are not secreted unless L chain mRNA is also injected. The finding that the selective inhibition of λ2 production in MOPC 315 by idiotype-specific T suppressor cells does not result in the secretion of free α chain (27) is also in agreement with our conclusion.

Genetic approaches have also implicated control at the level of assembly for secretion of J chain, the joining protein necessary for multimeric Ig protein formation. MOPC 315 mutants, which have lost the ability to synthesize α H chain, fail to secrete J chain protein (20). Only L chains can, in general, be secreted in the absence of H chains (2, 3, 6, 25). Some unusual L chains, the PC-specific chain (28) (Fig. 4) and the κ chain of myeloma tumor MOPC 21 (5), are secreted only when paired with a H chain. Their failure to be secreted presumably reflects some molecular feature of the variable region, a hypothesis consistent with the observation of Wu et al. (10), that the substitution in position 15 from gly to arg in the λ2 L chain of MOPC 315 blocks its secretion. It is interesting that the PC-specific μ and κ chains can be secreted together as IgM, whereas neither of these Ig chains can be secreted alone without the other.

The expression of the introduced λ2 L chain gene was very low, even though many copies (≥100) of the cloned DNA were detected in the LV-1 transfectants by Southern blot analysis (data not shown). This expression was probably driven by the SV40 enhancer, present on the pSV2-gpt vector, as was shown previously for transfection of the λ1 L chain gene (29). Alternatively, integration into a "transcriptionally open" site in the chromatin may have played a role. The inability of researchers to find a λ L

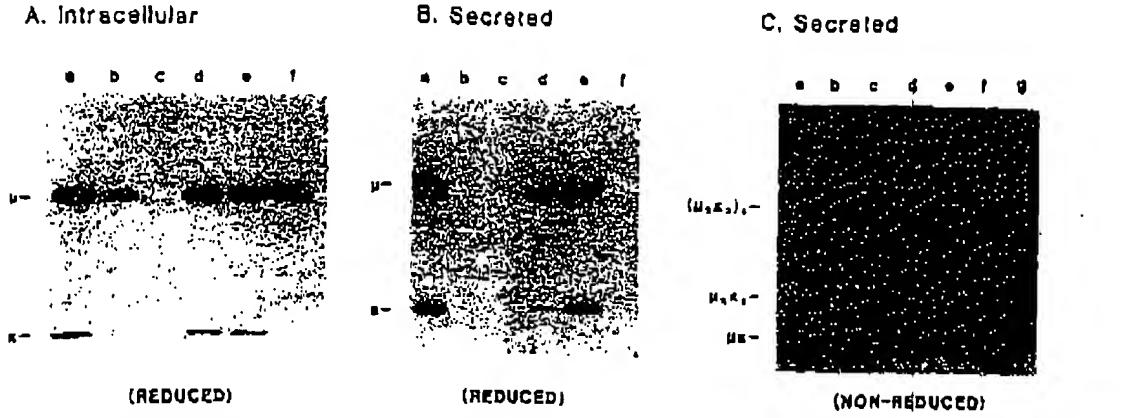

Figure 6. Analysis of Ig production after transfer of ε gene. The plasmid pNeo-εPc (Fig. 1b) was introduced into the bacterium K803, and protoplasts were fused with the mutant S74 myeloma cells. Colonies (transformants) resistant to 1 mg/ml G418 were selected and cloned by limiting dilution. Cells were incubated in [^{14}C]leucine, as described (4), to label intracellular and secreted Ig. The μ and ε chains were precipitated by reacting with a mixture of rabbit anti-μ and anti-ε sera. Lanes: a) wild-type PC7; b) mutant S74; c) mutant 400; d) transformant TS74/εpc-2; e) transformant TS74/εpc-10; f) transformant TS74/εpc-11. A. Analysis of intracellular material after reduction of disulfide bonds. B. Analysis of secreted material after reduction of disulfide bonds. C. Analysis of secreted material without reduction of disulfide bonds. In lane g, the mutant 21, which makes monomeric IgM (4), is included for comparison.

chain enhancer is certainly puzzling. Clearly in our experiments, the endogenous gene was expressed at a significantly higher rate than the introduced cloned version. Because the pGpt-λ2 clone encompassed 6.6 kb of the MOPC 315 λ2 L chain gene, this result suggests either that the regulatory sequences are more distant or that the endogenous gene has a different conformation, one that is better able to interact with cellular factors required for optimal λ L chain gene transcription.

Thus in a normal situation, free H chain polypeptide is not in an appropriate form to be secreted. This may be due simply to structural differences between a free H chain compared with a H chain assembled in an Ig molecule. Thorens et al. (30) have shown that free μ chains are directed via the *cis* golgi to lysosomes where they are degraded, whereas μ-L chain pairs migrate via the *trans* golgi to the cell surface. In some myeloma variants the H chain is degraded intracellularly (6); in other cases it remains within the cell (3). The results of the experiments presented here indicate that assembly of the H chain with a L chain is sufficient to restore secretion of those polypeptides in a complete Ig protein. The importance of assembly in control of secretion has also been demonstrated for collagen (31), β_2-microglobulin (32), and the T cell receptor (33). Thus, the assembly of multi-subunit proteins is often a requirement for protein secretion, and thus might serve as an important regulatory step in the secretory process.

Acknowledgments. We thank H. Murialdo for generously providing the pGpt-λ2 clone. The excellent typing of Ms. Michaelle Montgomery is gratefully acknowledged.

REFERENCES

1. Winkelhake, J. 1978. Immunoglobulin structure and effector functions. *J. Immunochem. 15:695.*
2. Edelman, G. M. 1973. Antibody structure and molecular immunology. *Science 180:830.*
3. Morrison, S. L., and M. Scharff. 1981. Mutational events in mouse myeloma cells. *CRC Crit. Rev. Immunol. 3:1.*
4. Shulman, M. J., C. Heusser, D. Filkin, and G. Kohler. 1982. Mutations affecting the structure and function of immunoglobulin M. *Mol. Cell. Biol. 2:1093.*
5. Kohler, G., S. C. Howe, and C. Milstein. 1976. Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. *Eur. J. Immunol. 6:292.*
6. Sonenshein, G. E., M. Siekevitz, G. Siebert, and M. L. Gefter. 1978. Control of immunoglobulin secretion in the murine plasmacytoma line MOPC 315. *J. Exp. Med. 148:301.*
7. Peale, P., M. Dean, V. Pepe, and G. E. Sonenshein. 1984. Overproduction of immunoglobulin mRNA by a murine myeloma MOPC 315 variant cell line. *J. Immunol. 133:478.*
8. Kohler, G., H. Rengartner, and M. J. Shulman. 1978. Immunoglobulin production by lymphocyte hybridomas. *Eur. J. Immunol. 8:82.*
9. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:680.*
10. Wu, G. E., N. Covinsky, N. Hozumi, and H. Murialdo. 1982. Nucleotide sequence of a chromosomal rearranged λ2 immunoglobulin gene of mouse. *Nucleic Acids Res. 10:3831.*
11. Mulligan, R. C., and P. Berg. 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA 78:2072.*
12. Kwan, S. P., S. Rudikoff, J. Seidman, P. Leder, and M. Scharff. 1981. Nucleic acid and protein sequences of phosphocholine-binding light chains. *J. Exp. Med. 153:1366.*
13. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet. 1:327.*
14. Sandri-Goldin, R. M., A. L. Goldin, M. Levine, and J. C. Glorioso. 1981. High-frequency transfer of cloned herpes simplex virus type 1 sequences to mammalian cells by protoplast fusion. *Mol. Cell. Biol. 1:743.*
15. Oshi, A., R. G. Hawley, M. J. Shulman, and N. Hozumi. 1983. Transfer of a cloned immunoglobulin light chain gene to mutant hybridoma cells restores specific antibody production. *Nature 302:340.*
16. Lynch, R. G., R. J. Graff, S. Sirisinha, E. S. Simms, and H. Eisen. 1972. Myeloma protein as tumor-specific transplantation antigens. *Proc. Natl. Acad. Sci. USA 69:1540.*
17. Peale, P., M. Siekevitz, R. Schwartz, M. Gefter, and G. E. Sonenshein. 1981. Transcription of immunoglobulin heavy chain sequences from the excluded allele. *Nature 291:594.*
18. Dean, M., R. Kent, and G. Sonenshein. 1983. Transcriptional activation of immunoglobulin α heavy chain genes by translocation of the c-myc oncogene. *Nature 305:443.*
19. Della Corte, E., and R. M. Parkhouse. 1973. Biosynthesis of immunoglobulin A (IgA). *Biochem. J. 136:589.*
20. Mosman, T., and R. J. Baumal. 1975. Synthesis but not secretion of J chain by variant mouse myeloma cells which lose α-chain-synthesizing ability. *J. Immunol. 115:955.*
21. Koshland, M. E. 1985. The coming of age of J chain. *Annu. Rev. Immunol. 3:425.*
22. Bergtelid, A., P. Perlman, and M. Scharff. 1972. Synthesis, assembly, and secretion of γ-globulin by mouse myeloma cells. IV. Assembly of IgA. *J. Immunol. 108:126.*
23. Gearhart, P., N. Johnson, R. Douglas, and L. Hood. 1981. IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. *Nature 291:29.*
24. Gronowicz, E., A. Coutinho, and F. Melchers. 1976. A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol. 6:588.*
25. Kohler, G. 1980. Immunoglobulin chain loss in hybridoma lines.

Proc. Natl. Acad. Sci. USA 77:2197.

26. Valle, G., J. Besley, and A. Colman. 1981. Synthesis and secretion of mouse immunoglobulin chains from Xenopus oocytes. *Nature 291:338.*
27. Parslow, T. G., G. Milburn, R. Lynch, and D. Granner. 1983. Suppressor T cell action inhibits the expression of an excluded immunoglobulin gene. *Science 220:1389.*
28. Ot, V. T., S. L. Morrison, L. A. Herzenberg, and P. Berg. 1983. Immunoglobulin gene expression in transformed lymphoid cells. *Proc. Natl. Acad. Sci. USA 80:825.*
29. Picard, D., and W. Schaffner. 1983. Correct transcription of a cloned mouse immunoglobulin gene in vivo. *Proc. Natl. Acad. Sci. USA 80:417.*
30. Thorens, B., M.-F. Schulz, and P. Vassalli. 1985. Bone marrow pre-B lymphocytes synthesize immunoglobulin μ-chains of membrane type with different properties and intracellular pathways. *EMBO J. 4:361.*
31. Rosenbloom, J., R. Endo, and M. Harsch. 1976. Termination of procollagen chain synthesis by puromycin. *J. Biol. Chem. 251:2070.*
32. Krangel, M. S., H. Orr, and J. Strominger. 1979. Assembly and maturation of HLA-A and HLA-B antigens in vivo. *Cell 18:979.*
33. Ghazal, P., T. W. Mak, P. Van den Elsen, Y. Yanagi, T. Yoshikai, A. Calman, C. Terhorst, J. Stobo, and A. Weiss. 1985. *Nature 316:606.*